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*P<sub>i</sub> release limits the intrinsic and RNA-stimulated ATPase cycles of DEAD-box protein 5 (Dbp5)*

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**ABSTRACT**

mRNA export from the nucleus depends on the ATPase activity of the DEAD-box protein Dbp5/DDX19. Although Dbp5 has measurable ATPase activity alone, several regulatory factors (e.g. RNA, nucleoporin proteins, and the endogenous small molecule InsP<sub>6</sub>) modulate catalytic activity *in vitro* and *in vivo* to facilitate mRNA export. To define how these factors control Dbp5 and mRNA export, an analysis of the intrinsic and regulator-activated Dbp5 ATPase cycle is necessary. Here, we report a kinetic and equilibrium analysis of the *Saccharomyces cerevisiae* Dbp5 ATPase cycle, including the influence of RNA on Dbp5 activity. These data show that ATP binds Dbp5 weakly in rapid equilibrium with a binding affinity ( $K_T \sim 4$  mM) comparable to the  $K_M$  for steady-state cycling, while ADP binds an order of magnitude more tightly ( $K_D \sim 0.4$  mM). The overall intrinsic steady-state cycling rate constant ( $k_{cat}$ ) is limited by slow, near-irreversible ATP hydrolysis and even slower subsequent phosphate release. RNA increases  $k_{cat}$  and rate-limiting  $P_i$  release 20-fold, although  $P_i$  release continues to limit steady-state cycling in the presence of RNA, in conjunction with RNA binding. Together this work identifies RNA binding and  $P_i$  release as important biochemical transitions within the Dbp5 ATPase cycle and provides a framework for investigating the means by which Dbp5 and mRNA export is modulated by regulatory factors.

## INTRODUCTION

DEAD-box proteins (DBPs) are ATPase enzymes central to RNA metabolism in all domains of life.<sup>1-3</sup> Many DBPs function as *bona fide* helicases that unwind duplex RNA, while others are involved in RNA-protein complex remodeling,<sup>4-6</sup> RNA folding,<sup>7, 8</sup> or serve as RNA sensors within the cell.<sup>9, 10</sup> DBPs accomplish this enormous diversity in function using a highly conserved catalytic core that couples ATP hydrolysis to nucleic acid binding and release.<sup>3, 8, 11, 12</sup> Consequently, DBPs are presumed to perform work via a common minimal enzymatic scheme involving ATP binding, hydrolysis, and subsequent phosphate ( $P_i$ ) and ADP product release.<sup>8, 11, 13, 14</sup>

The functional diversity of DBPs is thought to stem in part from kinetic and thermodynamic adaptations of this common ATPase cycling scheme.<sup>8</sup> For example, DbpA and Mss116 share a common RNA-activated ATPase cycle pathway, but variations in the catalytic cycle rate and equilibrium constants uniquely alter their interaction with RNA, which may allow them to fulfill specific biological functions.<sup>8, 13-15</sup> This adaptive behavior is analogous to the duty ratios of cytoskeleton motor proteins and demonstrates how kinetic modulation broadens the biological activities of an enzyme family.<sup>8, 16</sup>

The inherent ATPase cycles of some DBPs are additionally regulated through ancillary domains that mediate interactions with regulatory factors that can include RNAs and/or proteins. For example, Dbp5 is a DBP required for nuclear mRNA export that is stimulated by RNA and further regulated by two nucleoporin proteins (Gle1 and Nup159) and an endogenous small molecule (inositol hexakisphosphate or  $\text{InsP}_6$ ).<sup>17-28</sup> While it is clear that all components have global *in vitro* and *in vivo* effects on Dbp5 activity, there is not yet a consensus on the individual and concerted effects of these factors at a mechanistic level. Critical to deciphering the effect of

regulators on the ATPase cycle of Dbp5, or any DBP, is a quantitative characterization of the intrinsic ATPase cycle, as it is only with this base knowledge that we can undertake future in-depth studies on the mechanism of regulation by trans-activating factors and apply this to understanding the *in vivo* cellular function of DBPs. Deducing how trans-activating factors modulate the ATPase cycle of individual DBPs and the functions that emerge from this regulation will also further our understanding of this important class of enzymes as a whole, which function in almost all aspects of RNA biology.

Here, we have determined the minimal ATPase cycle and calculated the rate and equilibrium constants of individual biochemical transitions for Dbp5. This work has identified slow ATP hydrolysis and even slower subsequent phosphate release as key bottlenecks in the ATPase cycle of Dbp5 that function as targets for modulation by RNA and other regulatory cofactors. We also show that the fluorescent nucleotides *N*-methylantraniloyl ATP and ADP (mantATP, mantADP), common tools for kinetic studies of ATPases,<sup>29-33</sup> significantly alter the ATPase cycle rate and equilibrium constants of Dbp5 (Supplementary Information). Although mant-labeled nucleotides have been found to alter the catalytic cycles of other molecular motors, this is the first example to our knowledge of such significant mant fluorophore-induced alterations in DBP behavior, and highlights the ongoing need for system-specific controls regarding fluorescent labels.<sup>33-36</sup>

Having defined the minimal catalytic ATPase cycle of Dbp5, we show that in the presence of RNA phosphate release remains a limiting step in the hydrolysis cycle of Dbp5. However, we also observe evidence of a new RNA binding-associated rate-limiting step in the kinetic scheme of Dbp5, which identifies an additional feature in the minimal kinetic scheme of RNA-stimulated ATPase activity. Overall, by understanding the steps that limit Dbp5 function in

various biochemical states (e.g. RNA-bound vs. free) we are building a framework for understanding Dbp5 function *in vivo*, which may vary for the sub-cellular pools of Dbp5 and depend upon different regulators given the various functions proposed for these populations of Dbp5.<sup>17-20, 37-42</sup>

## RESULTS

### A. Intrinsic ATPase activity of Dbp5

#### *Steady state ATPase measurements*

Dbp5 has intrinsic ATPase activity in the absence of RNA or other regulatory factors.<sup>18, 20, 22</sup> As an essential starting point for quantitative analysis of the ATPase cycle of Dbp5, we first determined the maximal per-enzyme cycling rate, or  $k_{\text{cat}}$  (in units of  $\text{s}^{-1}$ ). Under our experimental conditions using both the NADH-coupled assay to detect ADP product formation and  $\text{P}_i\text{BiP}$  to detect phosphate product accumulation,<sup>43, 44</sup>  $k_{\text{cat}}$  is 0.04-0.08  $\text{s}^{-1}$  in the presence of saturating ATP (Fig. 1, Table 1). The  $K_M$  for ATP substrate ( $K_{M,\text{ATP}}$ ) is 1.3-1.9 mM (Fig. 1, Table 1). These values are comparable to published values obtained under different solution conditions.<sup>22, 45</sup>

#### *Transient kinetic analysis of the Dbp5 ATPase cycle*

To define the rate limiting steps within the intrinsic Dbp5 ATPase cycle, a combination of kinetic assays were used to measure individual rate and equilibrium constants. As described below, binding and dissociation of unlabeled nucleotides (ATP and ADP) were measured by competition with mant-labeled nucleotides, and constants related to ATP hydrolysis and product release were measured with  $\text{P}_i\text{BiP}$  and oxygen isotope exchange assays.

*Dbp5 nucleotide binding and dissociation* – The Förster Resonance Energy Transfer (FRET)-based fluorescence enhancement associated with mant-labeled nucleotide binding has been successfully used as a spectroscopic probe to characterize the ATPase cycles of DNA<sup>33, 35, 46-49</sup> and RNA helicases,<sup>13, 14, 30</sup> myosin,<sup>50-54</sup> and kinesin<sup>32, 34, 55, 56</sup> motor proteins, as well as G-proteins,<sup>57-59</sup> with modest perturbations introduced by the mant moiety. We monitored mant-



labeled ATP and ADP binding to Dbp5 from changes in FRET between Dbp5 tryptophans and the mant fluorophore. Time courses of fluorescence enhancement after rapidly mixing mantATP with Dbp5 was described by a sum of three exponentials (Fig. 2a and 2b, Supplementary Information), while mantADP binding was described by a sum of two exponentials (Fig. 2c and 2d, Supplementary Information). The multi-exponential behavior did not arise from the mixed mant isomers (Supplementary Information) and was therefore modeled as two (mantADP) or three (mantATP) detectable and sequential biochemical transitions. In the case of mantATP, the [mantATP]-dependence of the three observed rate constants yields both forward and reverse rate constants ( $k_{+mTi}$  and  $k_{-mTi}$ ,  $i=1, 2, 3$ . Table 1, Supplementary Information). The mantATP binding affinity ( $K_{mT}$ ; the mantATP equivalent of  $K_T$  in Scheme 1, see Materials and Methods) determined from the ratio of  $k_{-mT1}/k_{+mT1}$  is 7  $\mu$ M (Table 1).

The *overall* mantATP binding affinity ( $K_{mT,overall}$ , a composite representing mantATP binding and successively populated transitions) is in the range of 1  $\mu$ M (Table 1), several orders of magnitude tighter than the  $K_M$  of unlabeled ATP ( $K_{M,ATP} = 1.3$ -1.7 mM; Table 1) or the ATP binding affinity (1-2 mM) determined by NMR<sup>45</sup> and this study (discussed below). The >240-fold difference between mantATP and ATP binding affinities suggests that the mant fluorophore affects nucleotide binding to Dbp5<sup>33, 35</sup>. To identify the molecular origins of potential perturbations introduced by the mant moiety, we determined the structure of Dbp5 with bound mant-nucleotide.

*RNA/mantADP•BeF<sub>3</sub>-bound Dbp5 is structurally similar to RNA/ADP•BeF<sub>3</sub>-bound Dbp5*

– The structure of Dbp5 with bound nucleotide has not been determined by X-ray crystallography, but the structure of Dbp5 with bound ATP analog (ADP•BeF<sub>3</sub>) and RNA has

been reported.<sup>27</sup> Therefore, we determined the structure of Dbp5 with bound mantADP•BeF<sub>3</sub> and RNA, to assess the influence of the mant moiety and to identify any structural perturbations in the complex.

The crystal structure of RNA/mantADP•BeF<sub>3</sub>-bound Dbp5, refined to a resolution 1.8 Å, reveals hydrophobic interactions between the mant moiety and three adjacent residues (Met110, Phe112, and Val181; Fig. 3a). Binding of mantADP buries 3,397 Å<sup>2</sup> of the solvent accessible surface area of Dbp5, with the mant moiety of the ligand accounting for 511 Å<sup>2</sup>. If we assume that such change in hydrophobic surface area provides  $-15 \pm 1.2 \text{ cal mol}^{-1} \text{ Å}^{-2}$  (Ref. <sup>60</sup>), the additional contacts made by the mant fluorophore could potentially provide a total of  $-7.6 \text{ kcal mol}^{-1}$  to the binding free energy. The mant moiety would therefore increase the ATP affinity from 6.4 mM ( $\Delta G^{\circ} = -3.0 \text{ kcal mol}^{-1}$ ) to 20 nM ( $\Delta G^{\circ} = -10.6 \text{ kcal mol}^{-1}$ ) and the ADP affinity from 360 μM ( $\Delta G^{\circ} = -4.8 \text{ kcal mol}^{-1}$ ) to 1 nM ( $\Delta G^{\circ} = -12.4 \text{ kcal mol}^{-1}$ ). Although these mant nucleotide binding constants are considerably tighter than observed (Table 1), it demonstrates that hydrophobic interactions with the mant moiety can account for the tighter binding affinity.

Superposition of RNA/mantADP•BeF<sub>3</sub>-bound Dbp5 onto RNA/ADP•BeF<sub>3</sub>-bound Dbp5 (PDB ID **3PEY**) reveals a similar overall structure of the ATPase and bound ligands ( $\text{rmsd}_{\text{C-}\alpha} = 0.1106$ ,  $\text{rmsd}_{\text{all-atom}} = 0.3939$ )<sup>27</sup>, suggesting that structural perturbations of Dbp5 and RNA introduced by the mant moiety are minimal (Figs. S4 and S5). One notable difference between the superimposed structures is the displacement of a set of eight water molecules found in the nucleotide-binding site of the ADP•BeF<sub>3</sub>-bound structure by the mant moiety (Fig. 3b), which may further contribute to the differences in mant nucleotide binding.

*Unlabeled nucleotide binding to Dbp5*– Kinetic competition between mantATP or mantADP and unlabeled ATP was performed to obtain parameters for unlabeled ATP binding and dissociation. ATP slows all observed rate constants associated with mantATP and mantADP binding (Fig. 4; Table 1), consistent with ATP binding in a rapid equilibrium ( $>300\text{ s}^{-1}$ ) and competing with mant-nucleotides for Dbp5 binding.<sup>51, 61</sup> Rapid ATP binding kinetics are beyond stopped flow detection limits ( $\sim 2\text{ ms}$ ), so we used the reduction in  $k_{\text{mT1,obs}}$  with increasing [ATP] to estimate an unlabeled ATP binding affinity ( $K_T$ ) of 6 mM (Fig. 4b, Eq. 13, Table 1). Competition with mantADP yields an ATP affinity of 3 mM (Fig. 4, Table 1). The comparable (i.e.  $< 2$ -fold difference) ATP binding affinities determined from competition with mantATP or mantADP suggests that mantATP turnover (i.e. hydrolysis, product release and any steady state ATPase activity) occurs much more slowly than initial binding of ATP and does not affect the measured  $K_T$  (see Supplementary Information).

Kinetic competition between mantADP and ADP demonstrates that Dbp5 binds ADP in rapid equilibrium, but  $\sim 10$ -fold more tightly than ATP ( $K_D = 360\text{ }\mu\text{M}$ , Table 1; Fig. 4), in agreement with published values ( $400\text{--}570\text{ }\mu\text{M}$ ).<sup>45</sup> Thus, mant-labeled nucleotides bind Dbp5 more tightly than their unlabeled counterparts (Table 1). The multiple observed phases in mant-nucleotide binding, particularly those of mantADP binding, may reflect isomerization steps facilitated by additional interactions between Dbp5 and the fluorophore. These isomerizations may contribute to high-affinity binding of mant-labeled nucleotides.

*Dbp5 ATP hydrolysis and phosphate release*– Fluorescently labeled phosphate binding protein ( $\text{P}_i\text{BiP}$ ) has been used to measure transient  $\text{P}_i$  release from DBPs and other molecular motor proteins.<sup>13, 14, 44, 53</sup> Its tight binding of  $\text{P}_i$  ( $K_d = 0.1\text{ }\mu\text{M}$ ) and rapid association rate constant

( $> 3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ) provides real-time fluorescent detection of transient and steady-state  $P_i$  release.<sup>62</sup> Time courses of  $P_i$ BiP fluorescence enhancement after rapidly mixing Dbp5 with ATP display multiple phases. The most prominent features are a lag followed by a linear phase (Fig. 5). The linear portion corresponds to steady-state ATPase cycling. The steady-state cycling parameters ( $k_{\text{cat}} = 0.08 \text{ s}^{-1}$ ,  $K_{\text{M,ATP}} = 1.3 \text{ mM}$ ) obtained from fitting the [ATP]-dependence of the turnover rate (i.e. the slope of the linear regime) to Eq. 10 agree well with those obtained with the NADH-coupled assay (Table 1), providing independent determination of the steady-state parameters (Table 1).

The lag phase is defined by an observed rate constant whose value depends hyperbolically on the [ATP], yielding an ATP binding affinity ( $K_T$ ) of 4 mM (Fig. 5, Eq. 15), comparable to the values obtained by competition with mantATP and mantADP (Fig. 4). The observed lag phase rate constant at saturating ATP ( $\lambda_{\infty}$ ) has a value of  $2 \text{ s}^{-1}$  and corresponds to the sum of the ATP hydrolysis and  $P_i$  release rate constants (Scheme 1) as follows:<sup>13, 14</sup>

$$\lambda_{\infty} = k_{+H} + k_{-H} + k_{-Pi} \quad \text{Equation 1.}$$

The intercept of the lag phase at zero ATP ( $\lambda_0$ ) is defined by:

$$\begin{aligned} \lambda_0 &= \frac{k_{-T}k_{-H} + k_{-T}k_{-Pi} + k_{+H}k_{-Pi}}{k_{-T}} \\ &= k_{-H} + k_{-Pi} + \frac{k_{+H}k_{-Pi}}{k_{-T}} \end{aligned} \quad \text{Equation 2}$$

and has a value of  $\sim 0.02 \text{ s}^{-1}$ , though this is subject to experimental uncertainty since it is close to the origin. When ATP dissociation is rapid ( $k_{-T} \gg$  other rate constants preceding ADP release in Scheme 1, a condition that is fulfilled since ATP binds in rapid equilibrium), the net contributions from the third term ( $k_{+H}k_{-Pi}/k_{-T}$ ) of Eq. 2 are negligible and  $\lambda_0$  simplifies to the sum of ATP resynthesis ( $k_{-H}$ ) and  $P_i$  release ( $k_{-Pi}$ ) rate constants:

$$\lambda_0 \sim k_{-H} + k_{-P_i} \quad \text{Equation 3}$$

Combining Eqs. 1 and 3 allows the observed lag phase rate constant to be expressed as:

$$\lambda_{\infty} \sim k_{+H} + \lambda_0 \quad \text{Equation 4.}$$

Given the values of  $\lambda_{\infty}$  ( $\sim 2 \text{ s}^{-1}$ ) and  $\lambda_0$  ( $\sim 0.02 \text{ s}^{-1}$ ), we conclude that ATP hydrolysis ( $k_{+H}$ ) dominates  $\lambda_{\infty}$  and occurs with a rate constant of  $\sim 2 \text{ s}^{-1}$  (Table 1). This and a steady state cycling  $k_{\text{cat}}$  of  $0.08 \text{ s}^{-1}$  indicate that ATP hydrolysis is not rate limiting, though it can contribute to the value of  $k_{\text{cat}}$ .<sup>13-15</sup>

*Oxygen isotope exchange* – Oxygen isotopic exchange experiments were performed to determine the ratio between reverse hydrolysis ( $k_{-H}$ ) and phosphate release ( $k_{-P_i}$ ). After hydrolysis, Dbp5 with bound ADP- $P_i$  will either release  $P_i$  or resynthesize ATP, depending on the relative rate constants for  $P_i$  release and reverse hydrolysis. In each ATP hydrolysis event, a water-derived oxygen is incorporated into the liberated  $P_i$  and there is a defined probability (depending on the solution enrichment of  $^{18}\text{O}$ ) that the water-derived oxygen is a heavy isotope. Therefore, the  $^{18}\text{O}$  isotope content in the released  $P_i$  product reveals the number of hydrolysis events that occurred before  $P_i$  release and determine the probability ratio of ATP resynthesis versus phosphate release.<sup>13, 15, 61, 63</sup> In the absence of significant reverse hydrolysis,  $P_i$  will have a single water-derived oxygen, but significant reverse hydrolysis will generate  $P_i$  with multiple water-derived oxygens due to multiple rounds of hydrolysis and resynthesis prior to  $P_i$  release.

The  $P_c$  value is determined by measuring the relative populations of singly and multiply-labeled  $^{18}\text{O}$  phosphates, and is defined by the ATP resynthesis and  $P_i$  release constants according to<sup>61, 63, 64</sup>:

$$P_c = \frac{k_{-H}}{k_{-H} + k_{-P_i}} \quad \text{Equation 5.}$$

The ratio of the  $^{18}\text{O}_2$ : $^{18}\text{O}_1$  species was  $0.0101 \pm 0.0009$  for hydrolysis by Dbp5 (see Materials and Methods for full description of data analysis). This ratio corresponds to a  $P_c$  value of  $0.0113 \pm 0.0026$  for hydrolysis in 48.5%  $^{18}\text{O}$ -water, indicating that  $P_i$  release occurs almost 100-fold faster than reverse hydrolysis ( $k_{-P_i}/k_{-H} = 99$ ) and little ATP resynthesis occurs during steady-state cycling. Consequently, the y-intercept of the lag phase rate constant  $\lambda_0$  (Eq. 2) is dominated by  $k_{-P_i}$  with a value of  $\sim 0.02 \text{ s}^{-1}$ , and a  $k_{-H}$  value of  $0.0002 \text{ s}^{-1}$  (Eq. 3; Table 1). Thus, Dbp5 hydrolysis is essentially irreversible despite slow  $P_i$  release. The determined value of the phosphate release rate constant of  $k_{-P_i} \sim 0.02 \text{ s}^{-1}$  is comparable to the steady state cycling rate, suggesting it or a transition preceding rapid  $P_i$  release limits Dbp5 cycling.

*P<sub>i</sub> rebinding during ATPase cycling* – To assess if  $P_i$  rebinding contributes to overall Dbp5 ATPase cycling and to estimate the  $P_i$  rebinding rate constant, we used the NADH assay ( $P_i$  release is irreversible in the  $P_i$ BiP assay due to  $P_i$ BiP and mop) to measure Dbp5 steady-state ATPase activity over a wide range of added free  $P_i$ . The Dbp5 ATPase activity is not affected by inclusion of  $P_i$  up to 10 mM, yielding an apparent  $K_{P_i,SS} = \frac{[P_i][HD]}{[HDP_i]}$  value  $>10 \text{ mM}$  during steady-state cycling and making  $P_i$  release essentially irreversible.

Although the steady-state distributions of the biochemical states are not identical to the equilibrium distributions, it is possible to relate this apparent  $K_{P_i,SS}$  to the true equilibrium constant ( $K_{P_i,eq} = \frac{k_{-P_i}}{k_{+P_i}}$ ) with consideration of reaction flux. During initial steady state cycling,

the reaction flux at every transition (Scheme 1) is constant and equal to the observed product release rate,  $v_0$  (Eq. 10). For  $P_i$  release and rebinding, this is given by the expression

$$k_{-P_i}[HDP_i] - k_{+P_i}[P_i][HD] = v_0 \leq [H]_T k_{cat} \quad \text{Equation 6.}$$

Dividing both sides of Eq. 6 by  $k_{+P_i}[HDP_i]$ , rearranging terms, and substituting in the steady-state and equilibrium definitions of  $K_{P_i}$  yields

$$K_{P_i,eq} = \frac{k_{-P_i}}{k_{+P_i}} = \frac{[P_i][HD]}{[HDP_i]} + \frac{v_0}{k_{+P_i}[HDP_i]} = K_{P_i,SS} + \frac{v_0}{k_{+P_i}[HDP_i]} > K_{P_i,SS} \quad \text{Equation 7,}$$

indicating that the affinity of  $P_i$  at equilibrium is always weaker than that in steady state.

Therefore, since  $K_{P_i,eq} > 10$  mM and  $k_{-P_i} \sim 0.02 \text{ s}^{-1}$ ,  $k_{+P_i}$  must be slower than  $2 \times 10^{-6} \text{ s}^{-1}$ . These data suggest that  $P_i$  rebinding by Dbp5 is negligible under our assay conditions and does not result in substantial product inhibition, even in the absence of the  $P_i$  mop.

### *Kinetic simulations from global fitting*

Our transient kinetic analysis indicates that the intrinsic ATPase cycle of Dbp5 features rapid, weak ATP binding, moderately slow hydrolysis, and rate-limiting  $P_i$  release followed by rapid ADP dissociation. To verify the experimentally determined rate and equilibrium constants, we globally fit ATPase time courses acquired with NADH and  $P_i$ BiP assays (Figs. 1 and 5) at different [Dbp5] and [ATP] to Scheme 1 using KinTek Explorer software. All the rate constants in Scheme 1 were unconstrained in global fitting with the exception of the following ratios: the ATP and ADP binding affinities (defined by the ratio of  $k_-/k_+$ ) were constrained to the experimentally determined values of 6 mM and 310  $\mu$ M, respectively, and  $P_i$  release was constrained to a value  $\sim 100$ -fold faster than reverse hydrolysis. Our only fully constrained rate constant was  $P_i$  rebinding, which was set at a near-zero value since  $P_i$  release from Dbp5 is

essentially irreversible during steady-state cycling (and is irreversible in the presence of  $P_i$ BiP). The fundamental rate constants obtained from global fitting (Table 2) agree well with the experimentally determined rate constants (Table 1) and account for the experimental time courses for various assays.

Global fits by KinTek Explorer closely match the experimental NADH assay data (Fig. 1) when  $P_i$  release is either partially or solely rate-limiting. The requirement for two partially rate-limiting steps (from the distinguishing lag phase in transient phosphate release assays; Fig. 5) eliminates scenarios in which hydrolysis does not also contribute to  $k_{cat}$ . In support of this scheme, simulations generated from global fitting outputs are very sensitive to changes in both ATP hydrolysis/resynthesis and  $P_i$  release, but insensitive to changes in ATP/ADP binding and dissociation provided that those steps are much faster than hydrolysis and  $P_i$  release. The best global fit of the NADH experimental data was achieved with ATP hydrolysis ( $k_{+H}$ ) at  $0.16\text{ s}^{-1}$ , ATP resynthesis ( $k_{-H}$ ) at  $6 \times 10^{-5}\text{ s}^{-1}$ , and  $P_i$  release ( $k_{-P_i}$ ) at  $0.06\text{ s}^{-1}$  with minimal phosphate rebinding ( $k_{+P_i} \sim 0$ ), thereby making  $P_i$  release the major determinant of  $k_{cat}$  (Table 2).

We also globally fitted transient  $P_i$  release data (Fig. 5) with thermodynamically constrained rapid ATP/ADP binding and dissociation, and a  $P_c$  value of 0.01. Independent of the steady-state NADH assay global fits by KinTek Explorer, the transient  $P_i$  release time courses are best globally fitted by a mechanism in which  $P_i$  release is the major determinant of  $k_{cat}$ : ATP hydrolysis ( $k_{+H}$ ) occurs at  $0.62\text{ s}^{-1}$ , ATP resynthesis ( $k_{-H}$ ) at  $0.001\text{ s}^{-1}$ , and  $P_i$  release ( $k_{-P_i}$ ) at  $0.1\text{ s}^{-1}$ . Constraining ATP hydrolysis ( $k_{+H}$ ) to  $0.16\text{ s}^{-1}$  (the best global fit output from the NADH assay data) yields simulation outputs that overlay with the  $P_i$ BiP data (Table 2, Fig. 5). Once again, in all converged parameters,  $P_i$  release limits the  $k_{cat}$  of Dbp5.



The steady-state cycling parameters  $k_{cat}$  and  $K_{M,ATP}$  can also be calculated from fundamental rate constants, but the relationship depends on the kinetic scheme. Using a minimal 4-state model (Scheme 1), making no assumptions about the speed or reversibility of any of the associated transitions, the theoretical  $k_{cat}$  and  $K_{M,ATP}$  values are given by:<sup>13, 65</sup>

$$k_{cat} = \frac{k_{+H}k_{-Pi}k_{-D}}{(k_{+H} + k_{-H})(k_{+Pi} + k_{-D}) + k_{+H}k_{-Pi} + k_{-Pi}k_{-D}} \quad \text{Equation 8}$$

$$K_{M,ATP} = \frac{k_{-T}(k_{-H}(k_{+Pi} + k_{-D}) + k_{-Pi}k_{-D}) + k_{+H}k_{-Pi}k_{-D}}{k_T((k_{+H} + k_{-H})(k_{+Pi} + k_{-D}) + k_{+H}k_{-Pi} + k_{-Pi}k_{-D})} \quad \text{Equation 9}$$

Substituting the rate constants derived from global fitting of either steady-state NADH or P<sub>i</sub>BiP assays (Table 2) into the above equations yield a  $k_{cat}$  of 0.04 s<sup>-1</sup> and a  $K_{M,ATP}$  of 1.1 mM, which closely match our experimental steady-state data (0.04-0.08 s<sup>-1</sup> and 1.3-1.5 mM, respectively; Table 1).

In summary, the rate constants determined from globally fitting our experimental data to Scheme 1 (Table 2) are consistent with those determined experimentally (Table 1). Moreover, the global enzyme cycling parameters,  $k_{cat}$  and  $K_M$ , calculated using individual rate constants obtained from global fitting are comparable to the values determined experimentally. The high degree of agreement between experimentally-derived parameters (both steady-state ATPase and transient kinetics) and global fitting outputs demonstrates the robustness of the proposed kinetic scheme and the experimentally determined parameters. As such, the minimal ATPase scheme used here to model Dbp5 activity is sufficient to account for all observed transitions, and serves as a quantitative framework for evaluating the influence of RNA and regulatory factors.

## B. Alteration of the intrinsic Dbp5 ATPase cycle by RNA

### *P<sub>i</sub> release in the presence of RNA*

RNA significantly impacts the Dbp5 ATPase cycle.<sup>22</sup> To quantify these changes within the framework described thus far, we performed steady-state ATPase assays with Dbp5 in the presence of saturating levels of ATP (20 mM) and varying RNA (up to 7 mM), which resulted in a ~20- fold acceleration of  $k_{\text{cat}}$  ( $0.92 \pm 0.08 \text{ s}^{-1}$ ), confirming earlier studies (Fig. 6, Table 1).<sup>22</sup> Given that intrinsic Dbp5 ATPase cycling is mainly limited by  $P_i$  release, RNA must minimally accelerate  $P_i$  release 20-fold to achieve this increase in  $k_{\text{cat}}$ . We therefore evaluated if RNA had the predicted effect on  $P_i$  release, and also whether or not our modeled rate constants from Scheme 1 were predictive of overall intrinsic Dbp5 steady-state cycling.

$P_i$  release is primarily rate-limiting in the absence of RNA and other cofactors. Therefore, the majority of Dbp5 populates the ADP- $P_i$  state under *in vitro* steady-state cycling conditions (Fig. 7). We took advantage of this Dbp5-ADP- $P_i$  accumulation in a series of dual-mixing experiments to test the effects of RNA on  $P_i$  release from the pre-formed Dbp5-ADP- $P_i$  state. Dbp5 was first rapidly mixed with ATP and “aged” for various times to allow biochemical intermediates along the ATPase cycle to form, then rapidly mixed again with a range of [RNA] while monitoring  $P_i$  release with  $P_i$ BiP. Based on the intrinsic rates determined above, delay times of 0.05 s, 6 s, and 25 s should result in progressive Dbp5 population of ATP-bound (pre-hydrolysis), ADP- $P_i$  -bound (post-hydrolysis), and ADP-bound (post  $P_i$  release) states, respectively.

Delay times  $\geq 6 \text{ s}$  abolished the lag phase in  $P_i$ BiP fluorescence time courses with Dbp5 alone (i.e. no RNA; Fig 8a). This observation is consistent with formation of the post-hydrolysis

ADP- $P_i$  state during aging, thereby bypassing (at least) one of the transitions contributing to the lag phase observed in single mixing experiments (Fig. 5). Furthermore, a rapid burst of  $P_i$ BiP fluorescence was observed with longer delay times (25 s, Fig. 8a), consistent with  $P_i$ BiP binding to  $P_i$  liberated by Dbp5 via hydrolysis events that occurred during aging. From these data, we can conclude that our modeled rate constants for intrinsic Dbp5 cycling are predictive of actual Dbp5 behavior and that these dual-mixing experiments can be used to interrogate the effect of RNA on  $P_i$  release.

Upon addition of RNA in the second mix after aging, time courses of  $P_i$ BiP fluorescence display a lag phase followed by a linear phase (Fig. 8b), independent of the age time (from 0 to 25 s) even if the lag is absent from time courses with Dbp5 alone (e.g. 6 s age time). The lag phase observed rate constant with 10 mM RNA is  $\sim 4\text{--}6\text{ s}^{-1}$  for all aging periods examined. The lag phase observed rate constant also depends hyperbolically on [RNA], reaching a maximum value of  $\sim 5.5 (\pm 2)\text{ s}^{-1}$  at saturating [RNA] (Fig. 8c). Thus, RNA alters the hydrolysis cycle of Dbp5 by introducing two or more new rate-limiting steps, at least one of which does not depend on pre-population of a particular Dbp5-nucleotide bound state. This is most likely an RNA binding-dependent transition — either slow RNA binding or a slow isomerization following binding — which must occur prior to establishing a new steady-state.

The [RNA]-dependence of the lag (Fig. 8c) is presumably related to the RNA affinity ( $K_{d,RNA}$ ) of Dbp5 (with bound nucleotide) at sub-saturating [ATP], and its value of  $3 (\pm 3)\text{ mM}$  agrees well with the Michaelis constant  $K_{M,RNA}$ <sup>14</sup> of  $3.4 (\pm 0.8)\text{ mM}$  for [RNA] dependent Dbp5 ATPase obtained from steady-state NADH assays (Fig. 6b, Table 1). The lack of a  $P_i$  burst from the pre-formed Dbp5-ADP- $P_i$  state following RNA binding (delay times  $\geq 6\text{ s}$ , Fig. 8b) also establishes that  $P_i$  release must continue to be slow and partially rate-limiting, even when RNA is

present. However, we note that the dual-mixing experiments do not rule out the possibility that hydrolysis may also limit  $k_{\text{cat}}$  in the presence of RNA. We therefore conclude that at least two biochemical transitions — an isomerization associated with RNA binding, and  $P_i$  release — limit RNA-activated Dbp5 ATPase cycling.

#### *Oxygen isotope exchange in the presence of RNA*

RNA may also promote ATP hydrolysis by Dbp5, as with other DBPs.<sup>8, 66, 67</sup> A faster observed hydrolysis rate constant could result from accelerated ATP resynthesis, ATP hydrolysis, or both. The very slow rate of ATP resynthesis in the intrinsic Dbp5 ATPase cycle makes the first option a distinct possibility, and this is crucial to understanding the potential effects of other regulators. We therefore performed oxygen isotope exchange analysis of the RNA-activated, steady-state Dbp5 ATPase.

The  $P_c$  value in the presence of RNA is  $0.042 \pm 0.003$  (vs. 0.01 in the absence of RNA), which equates to a  $P_i$  release rate constant that is ~25-fold faster than ATP resynthesis (Table 1). Because  $P_i$  release is accelerated 20-fold in the presence of RNA, ATP resynthesis must also be accelerated to maintain the observed 4-fold difference in  $P_c$  value. Using the rate constants for  $P_i$  release, the  $P_c$  values and Eq. 5, we estimate that RNA accelerates ATP resynthesis by Dbp5 ~80-fold. Accelerating ATP resynthesis increases the observed ATP hydrolysis rate constant (i.e. shortens ATP hydrolysis relaxation time), as it is determined by the sum of forward and reverse rate constants.<sup>68</sup>

## DISCUSSION

### *General features of the intrinsic Dbp5 ATPase cycle*

Dbp5 has a slow maximum turnover rate ( $k_{\text{cat}} = 0.04 \text{ s}^{-1}$ ) and a weak  $K_{\text{M,ATP}}$  (1.3-1.9 mM). The slow  $k_{\text{cat}}$  results from slow ATP hydrolysis coupled to even slower  $\text{P}_i$  release, both of which limit overall cycling, though  $\text{P}_i$  release is the major determinant. It is experimentally difficult to measure hydrolysis directly by chemical quench-flow,<sup>13, 66, 69</sup> given weak ATP binding to Dbp5 (i.e. saturation requires ~20 mM ATP, a small fraction of which will be hydrolyzed even with tens of micromolar Dbp5, making detection by thin layer chromatography, HPLC, and other conventional means technically challenging). However, isotope exchange experiments and the presence of the lag in  $\text{P}_i$ BiP fluorescence time courses provide sufficient evidence to implicate both hydrolysis and  $\text{P}_i$  release as slow and relatively irreversible transitions that limit Dbp5 ATPase cycling in the absence of RNA and other regulatory factors. Current structural and biochemical data support a role for protein regulators (i.e. Gle1-InsP<sub>6</sub> and Nup159) in facilitating enzyme recycling post-hydrolysis,<sup>25, 27</sup> which fits well with the kinetic bottlenecks identified here. Future studies will be required to investigate if these regulators function in this fashion and/or facilitate other transitions in the ATPase cycle that become limiting in the presence of RNA and trans-activating factors.

### *Interactions with mant-labeled nucleotides*

We found that mant-labeled nucleotides severely perturb the kinetics and thermodynamics of the Dbp5 ATPase cycle. The rate and equilibrium constants are affected by several orders of magnitude and the rate-limiting transitions for intrinsic cycling are also perturbed (Supplementary Information). This is the first example to our knowledge of such

dramatic differences in behaviors between native and mant-labeled nucleotides with the DBP family of enzymes. While the origin of this fluorophore effect is still unclear, additional hydrophobic interactions with the mant fluorophore (Fig. 3, Supplementary Information) are likely to contribute. It is intriguing to speculate if the ability of Dbp5 to discriminate between ATP and ATP-like molecules may serve some additional function *in vivo*, as was recently described for the DBP Ded1 in AMP sensing.<sup>70</sup> Certainly, it demonstrates that fluorophore effects are not uniform even between closely related enzymes and must be checked.<sup>33, 35, 48</sup>

### *General features of the Dbp5 RNA-stimulated ATPase cycle*

Dbp5 steady state ATPase cycling is accelerated 20-fold by RNA (Table 1). Consequently, the main rate limiting step in the intrinsic Dbp5 cycle —  $P_i$  release — must be accelerated by RNA at least 20-fold. Intriguingly, RNA binding ( $k_{HT+R} = 1\text{-}5\text{ s}^{-1}$ ) becomes partially rate-limiting, and together with  $P_i$  release ( $k_{-RPi} = 1\text{-}5\text{ s}^{-1}$ ), limits steady state Dbp5 ATPase cycling in the presence of RNA. This suggests the interaction time between Dbp5 and RNA is  $\geq 0.4\text{ s}$  ( $\tau_{RNA\text{-binding}} + \tau_{P_i\text{-release}} = \frac{1}{k_{HT+R}} + \frac{1}{k_{-RPi}} \geq \frac{1}{5\text{ s}^{-1}} + \frac{1}{5\text{ s}^{-1}} = 0.4\text{ s}$ ), which may be significant for the role of Dbp5 in mRNA export (see discussion below) and/or other cellular processes.

### *Comparison to other DBPs*

The intrinsic Dbp5 ATPase cycle appears very similar to that of other RNA helicases. Like Dbp5, both DbpA and Mss116 display unfavorable hydrolysis and fully or partially rate-limiting  $P_i$  release in the absence of RNA.<sup>13-15</sup> In the case of DbpA, hydrolysis is so unfavorable that it is undetectable in the absence of its RNA activator, the peptidyl-transferase center (PTC)

of the 23S ribosomal RNA.<sup>13, 15, 71</sup> Although the equilibrium constants for ATP hydrolysis ( $K_H$ ) and  $P_i$  release ( $K_{P_i}$ ) of intrinsic Dbp5 ATPase activity actually favor progression through the ATPase cycle, the slow rate constants associated with both transitions bottleneck the intrinsic cycle at similar positions (i.e. biochemical states) as with DbpA and Mss116 cycling. In this sense, Dbp5 appears to be typical of the DBP family.

Despite these similarities with other representative DEAD-box proteins, some unique features of Dbp5 should be noted. First is the extremely weak millimolar ATP binding affinity (~4 mM) as compared to sub-millimolar binding affinities for most other studied DBPs.<sup>13, 14</sup> As a result, little Dbp5-ATP is populated under physiological conditions,<sup>72, 73</sup> rather, free Dbp5, Dbp5-ADP- $P_i$  and Dbp5-ADP are favored (Fig. 7).

A second feature of the intrinsic ATPase cycle of Dbp5 is the slow reverse hydrolysis (ATP resynthesis) rate constant both in the presence and absence of RNA, which maintains Dbp5 in an ADP- $P_i$  state when coupled with even slower  $P_i$  release (Fig. 7). Although accelerated at least 20-fold with RNA,  $P_i$  release remains at least partially rate-limiting, such that Dbp5 significantly populates the ADP- $P_i$  state even in the presence of RNA. Formation of such a stabilized biochemical intermediate may promote distinct and/or high affinity interactions with RNAs important for *in vivo* function, as with other DBPs,<sup>10, 13-15</sup> which may be required for the proposed ribonucleoprotein (RNP) remodeling activity of Dbp5 at NPCs.<sup>74-76</sup>

Alternatively, it has been demonstrated that Dbp5 shuttles through the nucleus,<sup>19</sup> interacts with transcriptional machinery,<sup>39</sup> and travels with nuclear RNPs from the site of transcription to NPCs<sup>37</sup>. This latter activity may rely on Dbp5 acting as an ATP-dependent RNA clamp and remaining bound to an mRNA, similar to eIFAIII as part of the exon-junction complex.<sup>9</sup> Maintenance of Dbp5 in an RNA-bound state would allow Dbp5 to be loaded on to mRNPs in

the nucleus during mRNP biogenesis and subsequently released upon reaching the cytoplasmic side of an NPC where known regulators (i.e. Gle1 and Nup159) are located, resulting in spatial regulation of the Dbp5 ATPase cycle. Within such a model, completion of the ATPase cycle only upon exiting the nucleus would facilitate directional mRNP export by initiating disruption of the Dbp5-RNP scaffold and mRNP remodeling. mRNA export times are reported to be in the range of a few hundred milliseconds<sup>77-80</sup>, as such the ability of Dbp5 to remain bound to RNA for  $\geq 400$  milliseconds is consistent with a clamp model for Dbp5 in mRNA export. To distinguish amongst these possibilities related to mRNA export and to understand how Dbp5 functions in other cellular processes, it will be critical to continue to investigate how regulators individually, and in combination, alter the Dbp5 ATPase cycle.



## MATERIALS AND METHODS

### *Chemicals*

All chemical reagents were of the highest purity commercially available. Millipore MiliQ® water (filtered through 0.2 µm filter) or Sigma RNase-free water was used in all buffers. ATP (Sigma A7699, ≥ 99% purity assayed by HPLC, ≤ 0.1% inorganic phosphate) and ADP (Roche Molecular Biochemicals) concentrations were determined by absorbance using  $\epsilon_{259}$  of 15,400 M<sup>-1</sup> cm<sup>-1</sup>. Polyuridylic acid (polyU RNA) (Santa Cruz Biotechnology, SC-215733A) was dialyzed extensively against distilled deionized water and ethanol precipitated prior to use. RNA concentrations were determined by absorbance using  $\epsilon_{260}$  of 9660 M<sup>-1</sup> cm<sup>-1</sup> and are in units of nucleotides. Mant-labeled nucleotides (2' and 3' mixed isomer, mantATP and mantADP from Invitrogen and mantATP, mant-dATP and mant-dADP from Jena Biosciences) concentrations were determined by absorbance ( $\epsilon_{255} = 23,300$  M<sup>-1</sup> cm<sup>-1</sup>). One molar equivalent of MgCl<sub>2</sub> was added to all nucleotides immediately before use.

All assays were performed at 25 ± 0.1 °C in 30 mM HEPES, pH 7.5, 100 mM KCl, 2 mM MgCl<sub>2</sub>, and 2 mM DTT.

### *Protein Expression, Purification and Labeling*

N-terminal 6xHis tagged Dbp5 was expressed and purified from *Escherichia coli* as previously described<sup>81</sup>. The *E. coli* phosphate binding protein (P<sub>i</sub>BiP) was purified and labeled with MDCC (7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl) coumarin; Thermo Fisher Scientific Inc.) as described.<sup>44, 82</sup>

### *Protein Crystallization*

Dbp5 crystal structures used the construct  $\Delta 90$ Dbp5 (Dbp5 residues 91-482) instead of full-length Dbp5<sup>27</sup>, purified as described above. In preparation for crystallization,  $\Delta 90$ Dbp5 was dialyzed into 30 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM DTT, 0.25 mM InsP<sub>6</sub> in 10% glycerol as previously described.<sup>27</sup> The N-terminal 6xHis tag was cleaved overnight at 4 °C with tobacco etch virus (TEV) protease in 5 mM DTT. Cleaved  $\Delta 90$ Dbp5 was purified by nickel affinity chromatography with Ni<sup>2+</sup>-NTA agarose (Qiagen) followed by size exclusion chromatography over a Superdex 200 (10/300) GL column (GE Healthcare) in 10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.25 mM InsP<sub>6</sub> in 10% glycerol. Purified  $\Delta 90$ Dbp5 was concentrated after the addition of 5 mM MgCl<sub>2</sub>. The  $\Delta 90$ Dbp5-mantADP•BeF<sub>3</sub> complex was formed by mixing concentrated  $\Delta 90$ Dbp5 in solution with a 1.2:1 molar ratio of rU<sub>10</sub> RNA (IDT), incubating 15 min, and then mixing mantADP•BeF<sub>3</sub> (prepared in a 1:3:15 ratio of ADP:Be:F) to a final concentration of 1 mM, followed by another 15 min incubation. Crystallization of  $\Delta 90$ Dbp5-mantADP•BeF<sub>3</sub> was performed by hanging drop vapor diffusion at 18 °C by mixing 100 nL of the complex at a protein concentration of 16 mg mL<sup>-1</sup> with 100 nL of reservoir solution containing 200 mM Mg(NO<sub>3</sub>)<sub>2</sub> and 18% PEG 3350 and incubating 4 days over 100  $\mu$ L of reservoir solution in a sealed chamber. Crystals were transferred to a cryoprotectant solution containing 18% PEG 3350, 9 mM HEPES, pH 7.5, 180 mM Mg(NO<sub>3</sub>)<sub>2</sub>, 4.5% glycerol, 1 mM DTT, 90 mM NaCl, 0.25 mM InsP<sub>6</sub>, 4.5 mM MgCl<sub>2</sub> and 1 mM mantADP•BeF<sub>3</sub> (prepared in a 1:3:15 ration of ADP:Be:F), and flash frozen in liquid nitrogen.

#### *Crystallographic Data Collection, Structure Determination, and Refinement*

Data were collected at 100 K on the NECAT 24-ID-E beam line of the Advanced Photon Source at the Argonne National Laboratory and processed with HKL.<sup>83</sup> Crystals belonged to the

space group  $P2_12_12_1$ . The structure of  $\Delta 90\text{Dbp5-mantADP}\cdot\text{BeF}_3$  was determined by molecular replacement with Phaser using the entire Dbp5 subunit (PDB ID code **3PEY**) as a search model.<sup>84</sup> The atomic coordinates and temperature factors were refined with REFMAC5, initially with noncrystallographic symmetry restraints applied.<sup>85</sup> The atomic model was improved with cycles of model building with COOT<sup>86</sup> followed by positional refinement. Rigid-body motions were modeled with REFMAC5 in terms of TLS tensors for translation, libration, and screw-rotation.<sup>87</sup> Water molecules were added using an automated procedure in COOT and by visual inspection. A final round of refinement was done with PHENIX.<sup>88</sup> Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession code **5ELX**. See Table S2 for data collection and refinement statistics.

#### *Steady-state ATPase assays*

Time courses of Dbp5 (0.6-1  $\mu\text{M}$ ) steady state ATPase assayed by absorbance change at 340 nm using the NADH-coupled assay were acquired on a Perkin-Elmer Lambda 20 UV-Vis spectrophotometer thermostatted at 25 °C.<sup>13, 43, 66</sup> The [ATP]-dependence of the initial steady-state ATPase rate ( $v_0$ ) was fitted to the Michaelis-Menten equation:

$$v_0 = \frac{k_{cat}[H]_T[T]}{[T] + K_{M,ATP}} \quad \text{Equation 10}$$

where  $k_{cat}$  is the maximal turnover rate of Dbp5 at saturating ATP in units of  $\text{s}^{-1}$ ,  $[H]_T$  is the total Dbp5 concentration,  $K_{M,ATP}$  is the Michaelis constant for ATP, and  $[T]$  is the total [ATP]. The [ADP] under our conditions of 2 mM ATP is  $\sim 7 \mu\text{M}$ .<sup>43</sup> Steady-state ATPase assays were also performed using ATP $\gamma$ S as a substrate.

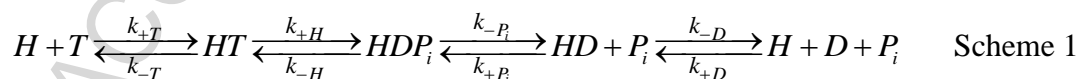
RNA-stimulated steady-state ATPase experiments were performed as detailed above, using 100 nM Dbp5 and 20 mM ATP. Data was fitted to the quadratic form of the Briggs-Haldane equation

$$v_{obs} = (k_{cat} - k_0) \frac{[H]_T + [R]_T - K_{M,RNA} - \sqrt{([H]_T + [R]_T - K_{M,RNA})^2 - 4[H]_T[R]_T}}{2[H]_T} + k_0 \quad \text{Equation 11}$$

where  $[R]_T$  is the total RNA concentration in nucleotides.<sup>66</sup>

### Transient kinetic assays

Transient kinetic measurements were done with an Applied Photophysics SX-2.0 stopped-flow apparatus thermostatted at  $25 \pm 0.1$  °C.<sup>13-15, 30, 66</sup> The time courses shown are either of raw, unaveraged traces or the average of 2-3 traces. The concentrations stated are final after mixing. Uncertainties are reported as standard errors in the fits unless stated otherwise. The following minimal reaction scheme with corresponding fundamental rate ( $k$ ) and equilibrium constants ( $K$ , defined by ratio of corresponding  $k$  values) was used for analysis and modeling:<sup>13-15, 66</sup>



where  $H$  is Dbp5,  $T$  is ATP,  $D$  is ADP, and  $P_i$  is inorganic phosphate. We assume product release is sequential with  $P_i$  preceding ADP. Several processes (e.g. nucleotide binding) are likely to be associated with multiple transitions (e.g. binding followed by an isomerization of the Dbp5-nucleotide complex), but are omitted for simplicity.<sup>8, 13-15, 66</sup>

### Nucleotide binding

Time courses of mant-nucleotide binding were measured by Förster resonance energy transfer from Dbp5 tryptophans to bound nucleotides. Fluorescence ( $\lambda_{\text{ex}} = 280 \text{ nm}$ ) was monitored through a 400 nm long pass colored filter. Inner filter effects from mant-nucleotide absorption are minimal at the mant-nucleotide concentrations used ( $< 120 \text{ } \mu\text{M}$ ). Time courses of association were acquired under both pseudo-first order and non-pseudo first order conditions. Data acquired under pseudo-first order conditions were fitted to a linear sum of exponentials. Time courses acquired under non-pseudo first order conditions were fitted with the following expression:<sup>89</sup>

$$F_0 + A_0 \left( 1 - \frac{r-1}{re^{-k_0t} - 1} e^{-k_0t} \right) + \sum_{i=1}^n A_i (1 - e^{-k_it}) \quad \text{Equation 12}$$

where  $F_0$  is the signal at time  $(t) = 0$ ,  $A_0$  and  $k_0$  are the signal amplitude and observed rate constant of the first phase, and  $r$  is an indication of the deviation of the bimolecular binding term (the second term in Eq. 12) from a single exponential. Under pseudo-first order conditions,  $r$  approaches 0 and the second term collapses to an exponential. When pseudo-first order conditions are not observed,  $r$  approaches 1, creating significant deviations from a single exponential.<sup>89</sup> The terms  $A_i$  and  $k_i$  ( $i = 1, \dots, n$ ) are the amplitudes and observed rate constants of additional phases following bimolecular binding, respectively. Irreversible mantADP dissociation was measured by rapidly mixing a pre-equilibrated mixture of  $1 \text{ } \mu\text{M}$  Dbp5 and  $10 \text{ } \mu\text{M}$  mantADP with  $1 \text{ mM}$  ADP and the time courses of fluorescence change were fitted to a sum of exponentials.<sup>51, 66</sup>

Unlabeled nucleotide binding was measured by kinetic competition with mant-labeled nucleotides.<sup>35, 51</sup> Time courses of fluorescence change associated with mant-nucleotide binding after rapidly mixing  $0.5\text{-}0.6 \text{ } \mu\text{M}$  Dbp5 with a mixture of  $20$  or  $50 \text{ } \mu\text{M}$  mant-nucleotide (either mantADP or mantATP) and varying concentrations of unlabeled nucleotide (ATP or ADP) were

fitted to a sum of exponentials. The unlabeled nucleotide binding affinity (dissociation equilibrium constant,  $K_d$ ) was determined from the [unlabeled nucleotide]-dependence of the observed mant-nucleotide binding rate constant, as defined by:<sup>51, 64</sup>

$$k_{obs} = \frac{(k_0 - k_{\infty})}{1 + \frac{[N]}{K_d}} + k_{\infty} \quad \text{Equation 13}$$

where  $[N]$  is unlabeled nucleotide concentration, and  $k_0$  is the observed mant-nucleotide rate constant in the absence of competing unlabeled nucleotide ( $[N] = 0$ ), and  $k_{\infty}$  at saturating  $[N]$ .

#### *Transient $P_i$ release of intrinsic Dbp5 ATPase*

Phosphate release from Dbp5 was assayed from the eightfold increase in fluorescence ( $\lambda_{ex} = 436$  nm, 463 nm long pass colored glass emission filter) of the MDCC-labeled  $P_i$ BiP upon binding phosphate. Contaminating  $P_i$  was removed from all equipment and buffers with a  $P_i$  “mop” consisting of 0.5 mM 7-methylguanosine and 0.01 U/mL purine nucleoside phosphorylase (Sigma). Since the process of mop’s scavenging free  $P_i$  in solution is very slow on the seconds time scale and  $P_i$ BiP binds  $P_i$  much faster (<100 ms) and extremely tightly, the presence of mop has minimal effect on transient and steady-state  $P_i$  release measurements, especially at short timescales.<sup>62</sup> Furthermore, 7-methylguanosine (up to 250  $\mu$ M) does not alter mantATP binding kinetics (data not shown).  $P_i$ BiP fluorescence was converted to  $[P_i]$  following calibration with phosphate standard solutions. Time courses of  $P_i$  liberation ( $[P_i]$ ) measured after rapidly mixing 0.5  $\mu$ M Dbp5 with an equilibrated mixture of 5  $\mu$ M of  $P_i$ BiP and varying ATP concentrations were fitted to the following expression defining a lag phase followed by a linear phase:<sup>13, 14</sup>

$$[P_i] = \beta \left( \frac{1}{\lambda} (e^{-\lambda} - 1) + t \right) + P_{i,0} \quad \text{Equation 14}$$

The linear regime represents steady-state ATPase, and its slope (given by  $\beta$ ) is the turnover rate.

The lag phase is defined by an exponential with observed rate constant  $\lambda$ . The [ATP]-

dependence of the observed lag phase rate constant was fitted to a hyperbola in the following form:<sup>13, 14</sup>

$$\lambda = \frac{(\lambda_{\infty} - \lambda_0)[T]}{K_T + [T]} + \lambda_0 \quad \text{Equation 15}$$

where [T] is the ATP concentration,  $\lambda_{\infty}$  is the observed lag phase rate constant at saturating ATP,  $\lambda_0$  is the intercept value at zero ATP, and  $K_T$  is the ATP dissociation equilibrium constant.

#### *RNA-stimulated transient $P_i$ release of Dbp5 ATPase*

Single-mix experiments were conducted above with the following modifications: 0.4  $\mu\text{M}$  Dbp5 was pre-equilibrated with varying concentrations of RNA, and rapidly mixed with 2.5 mM ATP pre-equilibrated with 5  $\mu\text{M}$   $P_i\text{BiP}$ .  $P_i$  mop was present in all solutions. Data was analyzed as detailed above.

Dual-mix experiments were conducted as above, with the modification that all solutions contained both 5  $\mu\text{M}$   $P_i\text{BiP}$  and  $P_i$  mop. Using the dual-mix mode of the SX20 stopped-flow, 5  $\mu\text{M}$  Dbp5 was mixed with 4 mM ATP and aged for 0.05, 6, or 25 s. After aging, the Dbp5/ATP solution was rapidly mixed with 0 or 10 mM RNA (nucleotide). Final concentrations were 2.5  $\mu\text{M}$  Dbp5, 2 mM ATP, 0 or 10 mM RNA, and 5  $\mu\text{M}$   $P_i\text{BiP}$ . Time courses of  $[P_i]$  release in the absence of RNA were fit to a sum of bimolecular binding and a linear function. Time courses of  $[P_i]$  in the presence of RNA were fit to the following sum of bimolecular binding and combined lag/linear phase:

$$[P_i] = F_0 + A_0 \left( 1 - \frac{r-1}{re^{-k_0t} - 1} e^{-k_0t} \right) + \beta \left( \frac{1}{\lambda} (e^{-\lambda t} - 1) + t \right) \quad \text{Equation 16.}$$

*Oxygen isotope exchange*

ATPase reactions (5-10  $\mu\text{M}$  Dbp5, 5-10 mM ATP) were conducted in 5-6 mM phosphoenolpyruvate (PEP), 50 U mL<sup>-1</sup> phosphoenolpyruvate kinase (PK), and 48.5% <sup>18</sup>O-labeled water. Reactions were run at room temperature for several hours to generate 2-3 mM P<sub>i</sub>, then quenched with an equivalent volume of 1 N HCl. P<sub>i</sub> was isolated and analyzed by coupled gas chromatography/mass spectrometry (GCMS).<sup>61, 64</sup> GCMS analysis of P<sub>i</sub> samples from hydrolysis of unlabeled ATP in <sup>18</sup>O-enriched water yields the distribution of species with zero to four <sup>18</sup>O-oxygens per P<sub>i</sub>. The  $P_c$  value was not determined by global fitting of this complete distribution because the starting reactions contained variable amounts of unenriched P<sub>i</sub> that result in an excess of the <sup>18</sup>O<sub>0</sub> species. At the low extents of reversal observed with Dbp5, most of the P<sub>i</sub> contains only one water-derived oxygen with only a small amount of P<sub>i</sub> that contains two water-derived oxygens. The amount of the species with two water-derived oxygens increases with increasing  $P_c$  value. The ratio of the <sup>18</sup>O<sub>2</sub>:<sup>18</sup>O<sub>1</sub> species with 2 versus 1 labeled oxygens was therefore used to determine  $P_c$  because this ratio only depends on the enrichment in the water and the  $P_c$  value and is uninfluenced by the amount of contaminating unenriched P<sub>i</sub>.

Oxygen isotope exchange experiments were also performed with RNA-stimulated Dbp5 ATPase activity. Steady-state reactions in the presence of RNA used 2  $\mu\text{M}$  Dbp5, 2.5 mM ATP, and 8 mM polyU RNA. Data was analyzed as above.

*Simulation of transient and steady-state activity*



Kinetic simulations by global fitting of both steady-state ATPase time courses acquired with the NADH assay and transient/steady-state  $P_i$  release acquired by  $P_i$ BiP fluorescence were done using Scheme 1 and KinTek Global Kinetic Explorer Software (KinTek Corp).<sup>90, 91</sup> A rapid ( $\gg k_{cat}$ ; e.g.  $\geq 10 \text{ s}^{-1}$ ) transition accounting for ATP regeneration from liberated ADP was also included to account for the activity of lactate dehydrogenase and phosphoenolpyruvate kinase in the NADH assay.

#### *Accession numbers*

Coordinates of the crystal structure of Dbp5 bound to RNA and mantADP•BeF<sub>3</sub> have been deposited in the Protein Data Bank under the accession number **5ELX**.

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Figure 1. Steady-state ATPase activity of Dbp5. (a) Time courses of absorbance change at 340 nm assayed with the NADH-coupled assay after mixing 660 nM Dbp5 with (lower to upper curves) 0, 0.25, 0.5, 1, 2, 8, or 20 mM ATP. The continuous lines through the data represent the best fits to linear functions, yielding the steady-state ATPase rates from the slopes. The dashed lines represent kinetic simulations of Scheme 1 and the corresponding rate and equilibrium constants obtained from global fitting with Kintek Explorer in Table 2. (b) [ATP]-dependence of the Dbp5 steady state ATPase rate. The continuous line through the data represents the best fit to a rectangular hyperbola, yielding the maximum velocity per enzyme ( $k_{cat}$ ) from the amplitude and the  $K_{M,ATP}$  from the [ATP] at half-maximum velocity (Table 1). Uncertainty bars represent standard errors in the fits and are within the data points.

Figure 2. mant-nucleotide binding to Dbp5. (a) Time courses of fluorescence change after mixing 0.5  $\mu$ M Dbp5 with (lower to upper) 0, 2.5, 5, 10, 20, 30, or 50  $\mu$ M mantATP. The smooth lines through the data represent the best fits to 3 exponentials. (b) Full time courses from (a) shown on a log scale to 200 s. (c) [mantATP]-dependence of the observed rate constants associated with mantATP binding. Continuous lines represent the best linear fits to the data. Uncertainty bars representing the standard errors of the best fits are within the data points. (d-f) Analogous mantADP binding data fitted to 2 exponentials shown on linear (d) and log (e) scales, and with rate constants fitted to hyperbolic or linear functions (f).

Figure 3. Interactions between mant-ADP•BeF<sub>3</sub> and Dbp5. (a) The mant fluorophore buries adjacent residues (Val181, M110, and Phe112) of  $\Delta 90$ Dbp5 *via* hydrophobic interactions ( $<4.0$  Å). The mant moiety hydrogen bonds (dashes) with waters at the solvent exposed surface. (b) Displacement of a network of hydrogen-bonded water molecules by the mant fluorophore.  $\Delta 90$ Dbp5-ADP•BeF<sub>3</sub> (blue) and the associated water molecules (red) of 3PEY superimposed to  $\Delta 90$ Dbp5-mant-ADP•BeF<sub>3</sub> and the associated water molecules (grey).  $\pi$ -stacking of the adenine is maintained. rmsd = 0.1106 Å.

Figure 4. Unlabeled nucleotide binding affinity measured by competition with mant nucleotides. (a) Time courses of fluorescence change after mixing 0.5  $\mu$ M Dbp5 with 50  $\mu$ M mantADP and 0, 0.175 mM, 0.75 mM, 2 mM, 5 mM, or 10 mM ADP (upper to lower). Continuous lines through the data represent best fits to 2 exponentials. (b) [nucleotide] dependence of the fastest observed rate constant ( $k_{1,obs}$ ) after mixing 0.5  $\mu$ M Dbp5 with a solution of 50  $\mu$ M mantADP supplemented with 0, 0.1, 0.3, 0.6, 1, 2.5, 5, or 11 mM ATP (black squares). Black circles represent the fastest observed rate constant of mantADP binding on mixing 0.5  $\mu$ M Dbp5 with a solution of 50  $\mu$ M mantADP supplemented with 0.005, 0.015, 0.03, 0.0625, 0.175, 0.75, 2, 5, or 10 mM ADP. Solid lines represent best fits to Eq. 13.

Figure 5. Transient and steady-state P<sub>i</sub> release during Dbp5 ATPase cycling. (a) Time courses of fluorescence change after mixing 0.5  $\mu$ M Dbp5 with a 5  $\mu$ M P<sub>i</sub>BiP containing (lower to upper curves) 0, 0.5, 1, 2, or 5 mM ATP. Fits to Eq. 14 are shown as dashed lines through the data.

Simulated data (using rate and equilibrium constants obtained from Kintek Explorer global fitting, Table 2) are shown as dotted lines through the data. (b) [ATP]-dependence of the observed  $P_i$  release lag phase rate constant ( $\lambda$ ). Continuous line through the data points represents best fit to a rectangular hyperbola (Eq. 15).

Figure 6. RNA-stimulated steady-state ATPase activity of Dbp5. Time courses of absorbance change at 340 nm assayed with the NADH-coupled assay after mixing 100 nM Dbp5 and 20 mM ATP with (lower to upper curves) 0, 0.45, 1.25, 2, 3, or 4.5 mM polyuridylic acid (concentration refers to total nucleotides). The continuous lines through the data represent the best fits to linear functions, yielding the steady-state ATPase rates from the slopes. (b) [ATP]-dependence of the RNA-stimulated Dbp5 steady state ATPase rate. The continuous line through the data represents the best fit to Eq. 11, yielding the maximum velocity per enzyme ( $k_{cat}$ ) from the amplitude and the  $K_{M,RNA}$  from the [ATP] at half-maximum velocity (Table 1). Uncertainty bars represent standard errors in the fits and are within the data points.

Figure 7. Steady-state distribution of Dbp5 ATPase cycle intermediates. *In vitro* conditions are 20 mM ATP, 0 mM ADP, 0 mM  $P_i$ . *In vivo* conditions are 2.1 mM ATP<sup>72</sup>, 470  $\mu$ M ADP<sup>72</sup>, 2.5 mM  $P_i$ <sup>73</sup>.

Figure 8. Transient RNA-stimulated  $P_i$  release during Dbp5 ATPase cycling. (a) Time courses of fluorescence change after mixing 5  $\mu$ M Dbp5 with 4 mM ATP and aging for (lower to upper) 0.05 s, 6 s, or 25 s prior to rapidly mixing with 5  $\mu$ M  $P_i$ BiP. Dashed lines are best fits to (lower to upper) Eq. 14, linear equation, or bimolecular binding plus a linear phase, respectively. (b) Time courses of fluorescence change after mixing 5  $\mu$ M Dbp5 with 4 mM ATP, aging for 6 s, and then rapidly mixing with 0 or 10 mM RNA in the presence of 5  $\mu$ M  $P_i$ BiP. Fits to Eq. 16 are shown as dashed lines through the data. (c) [RNA]-dependence of the observed  $P_i$  release lag phase rate constant ( $\lambda$ ) where pre-equilibrated Dbp5 and RNA are rapidly mixed with ATP and  $P_i$ BiP. Continuous line through the data points represents best fit to a rectangular hyperbola (Eq. 15), yielding a maximum observed rate constant of  $5.5 \pm 2 \text{ s}^{-1}$  upon extrapolation of the fit to saturating RNA. The [RNA] at half maximal is  $\sim 3 \text{ mM}$  under these non-saturating ATP concentrations.

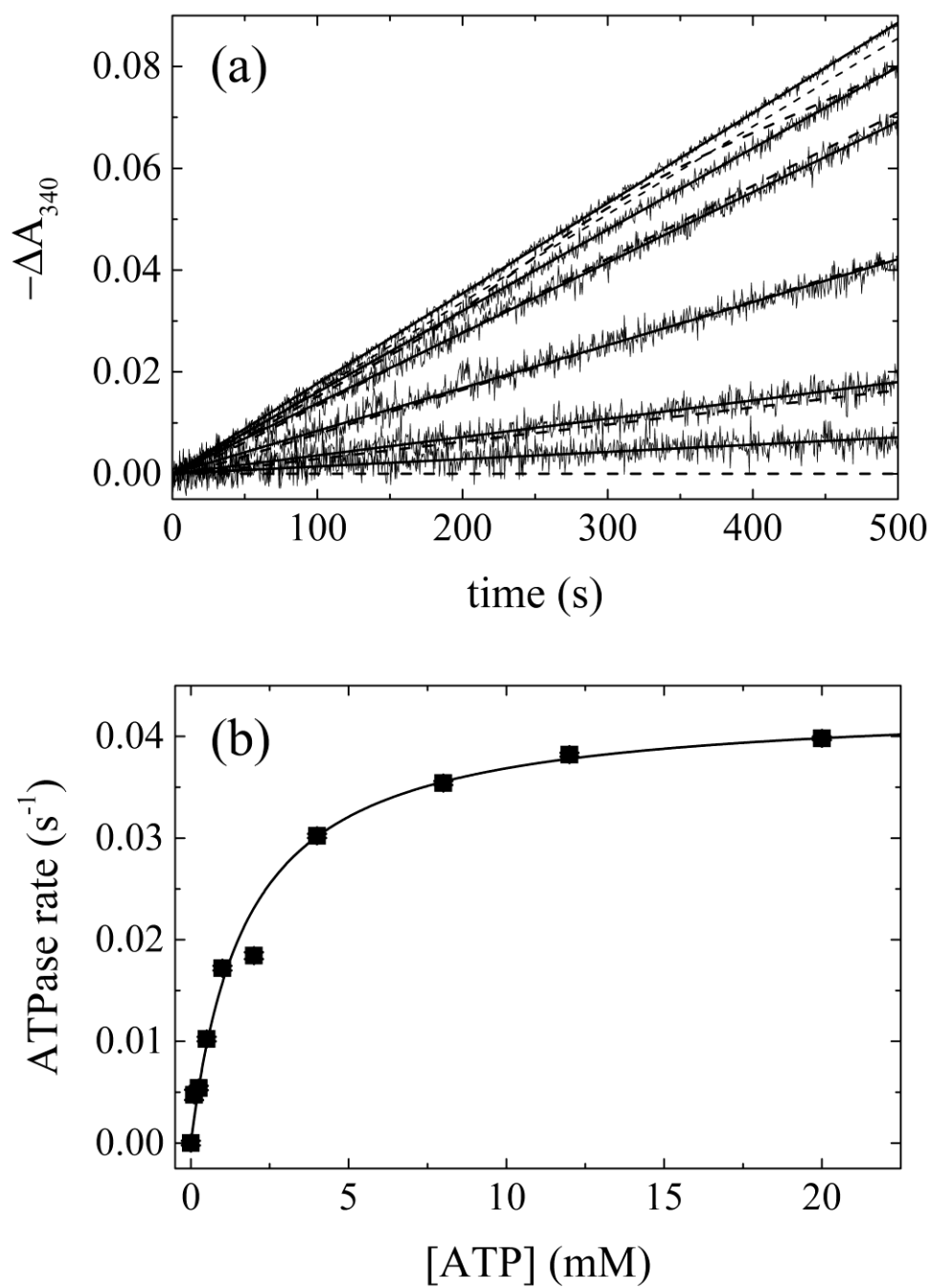
**Table 1. Rate and equilibrium constants of the Dbp5 ATPase cycle.**

Parameter	Value	Units	Assay
<i>Steady-state ATPase</i>			
$k_{\text{cat}}$	0.043 ( $\pm 0.001$ )	$\text{s}^{-1}$	NADH linked assay
	0.084 ( $\pm 0.002$ )	$\text{s}^{-1}$	P <sub>i</sub> BiP
$K_{\text{M,ATP}}$	1.7 ( $\pm 0.2$ )	mM	NADH linked assay
	1.3 ( $\pm 0.1$ )	mM	P <sub>i</sub> BiP
<i>Steady-state ATPase of ATP<math>\gamma</math>S</i>			
$k_{\text{cat}}$	0.036 ( $\pm 0.001$ )	$\text{s}^{-1}$	NADH linked assay
$K_{\text{M,ATP}\gamma\text{S}}$	0.98 ( $\pm 0.06$ )	mM	NADH linked assay
<i>ATP and mantATP binding</i>			
$k_{+\text{mT1}}$	1.63 ( $\pm 0.04$ )	$\mu\text{M}^{-1} \text{s}^{-1}$	mantATP
$k_{-\text{mT1}}$	11.9 ( $\pm 0.7$ )	$\text{s}^{-1}$	mantATP
$K_{\text{mT1}}$	7.3 ( $\pm 0.4$ )	$\mu\text{M}$	$k_{-}/k_{+}$
$k_{+\text{mT2}}$	0.4 ( $\pm 0.6$ )	$\text{s}^{-1}$	mantATP
$k_{-\text{mT2}}$	1.9 ( $\pm 0.4$ )	$\text{s}^{-1}$	mantATP
$K_{\text{mT2}}$	5 ( $\pm 7$ )		$k_{-}/k_{+}$
$k_{\text{mT3,obs}}$	0.01 ( $\pm 1 \times 10^{-4}$ )	$\text{s}^{-1}$	mantATP
$K_{\text{mT,overall}}$	1 ( $\pm 0.3$ )	$\mu\text{M}$	Total fluorescence amplitude
$K_{\text{T}}$	6.4 ( $\pm 0.7$ )	mM	mantATP competition
	3.0 ( $\pm 0.4$ )	mM	mantADP competition
	4 ( $\pm 2$ )	mM	P <sub>i</sub> BiP
<i>ATP hydrolysis</i>			
$k_{+\text{H}}$	2.2 ( $\pm 0.4$ )	$\text{s}^{-1}$	P <sub>i</sub> BiP
$k_{-\text{H}}$	$2 \times 10^{-4}$ ( $\pm 5 \times 10^{-5}$ )	$\text{s}^{-1}$	P <sub>i</sub> BiP & isotope exchange
$K_{\text{H}}$	$1 \times 10^{-4}$ ( $\pm 3 \times 10^{-5}$ )		$k_{-}/k_{+}$
<i>Phosphate release</i>			
$k_{-\text{Pi}}$	0.02 ( $\pm 0.1$ )	$\text{s}^{-1}$	P <sub>i</sub> BiP
$k_{+\text{Pi}}$	$< 2 \times 10^{-6}$	$\mu\text{M}^{-1} \text{s}^{-1}$	$k_{-\text{Pi}}/K_{\text{Pi}}$

$K_{P_i}$	10	mM	Steady-state $P_i$ inhibition
<i>ADP and mantADP binding</i>			
$K_{mD0}$	102 ( $\pm$ 21)	$\mu$ M	mantADP
$k_{+mD1}$	98 ( $\pm$ 15)	$s^{-1}$	mantADP
$k_{-mD1}$	0.2 ( $\pm$ 0.5)	$s^{-1}$	mantADP
	2.611 ( $\pm$ 0.003)	$s^{-1}$	Irreversible mantADP dissociation
$K_{mD1}$	0.002 ( $\pm$ 0.005)		$k_{-}/k_{+}$ mantADP
$k_{mD2,obs}$	$\sim$ 0.7 ( $\pm$ 0.1)	$s^{-1}$	mantADP
$K_D$	0.36 ( $\pm$ 0.05)	mM	mantADP competition
<i>RNA-stimulated steady-state ATPase activity</i>			
$k_{cat}$	0.92 ( $\pm$ 0.08)	$s^{-1}$	NADH linked assay
$K_{M,RNA}$	3.4 ( $\pm$ 0.8)	mM	NADH linked assay
<i>Oxygen Isotope exchange</i>			
$P_c$	0.0416 ( $\pm$ 0.0025)		GCMS

**Table 2. Rate and equilibrium constants determined from global fitting.**

Parameter	Value	Units	Assay
<i>Steady-state ATPase activity</i>			
$k_{\text{cat}}$	0.04 ( $\pm 0.0001$ )	$\text{s}^{-1}$	Eq. 8
$K_{\text{M,ATP}}$	1.4 ( $\pm 1.1$ )	mM	Eq. 9
<i>ATP binding</i>			
$k_{+T}$	0.92 ( $\pm 0.03$ )	$\mu\text{M}^{-1} \text{s}^{-1}$	KinTek steady-state simulation
$k_{-T}$	3670	$\text{s}^{-1}$	KinTek steady-state simulation
$K_T$	4	mM	Constrained from competition assay
<i>ATP hydrolysis</i>			
$k_{+H}$	0.16 ( $\pm 5 \times 10^{-4}$ )	$\text{s}^{-1}$	KinTek steady-state simulation
	0.72 ( $\pm 0.01$ )	$\text{s}^{-1}$	KinTek P <sub>i</sub> BiP simulation
$k_{-H}$	$6 \times 10^{-4}$ ( $\pm 8 \times 10^{-7}$ )	$\text{s}^{-1}$	KinTek steady-state simulation
	0.38 ( $\pm 0.05$ )	$\text{s}^{-1}$	KinTek P <sub>i</sub> BiP simulation
$K_H$	0.004 ( $\pm 5 \times 10^{-4}$ )		KinTek steady-state simulation
	0.53 ( $\pm 0.07$ )		KinTek P <sub>i</sub> BiP simulation
<i>Phosphate release</i>			
$k_{-P_i}$	0.062 (Constrained)	$\text{s}^{-1}$	KinTek steady-state simulation
	0.04 ( $\pm 0.02$ )	$\text{s}^{-1}$	KinTek P <sub>i</sub> BiP simulation
$k_{+P_i}$	$< 1 \times 10^{-6}$ (Constrained)	$\text{s}^{-1}$	KinTek steady-state simulation
	$< 1 \times 10^{-6}$	$\text{s}^{-1}$	KinTek P <sub>i</sub> BiP simulation
$K_{P_i}$	$> 1 \times 10^{-4}$	$\mu\text{M}$	KinTek steady-state simulation
	$> 1 \times 10^{-4}$	$\mu\text{M}$	KinTek P <sub>i</sub> BiP simulation
<i>ADP release</i>			
$k_{+D}$	0.2	$\mu\text{M}^{-1} \text{s}^{-1}$	KinTek steady-state simulation
$k_{-D}$	64 ( $\pm 16$ )	$\text{s}^{-1}$	KinTek steady-state simulation
$K_D$	0.31	mM	Constrained from competition assay

**Figure 1**



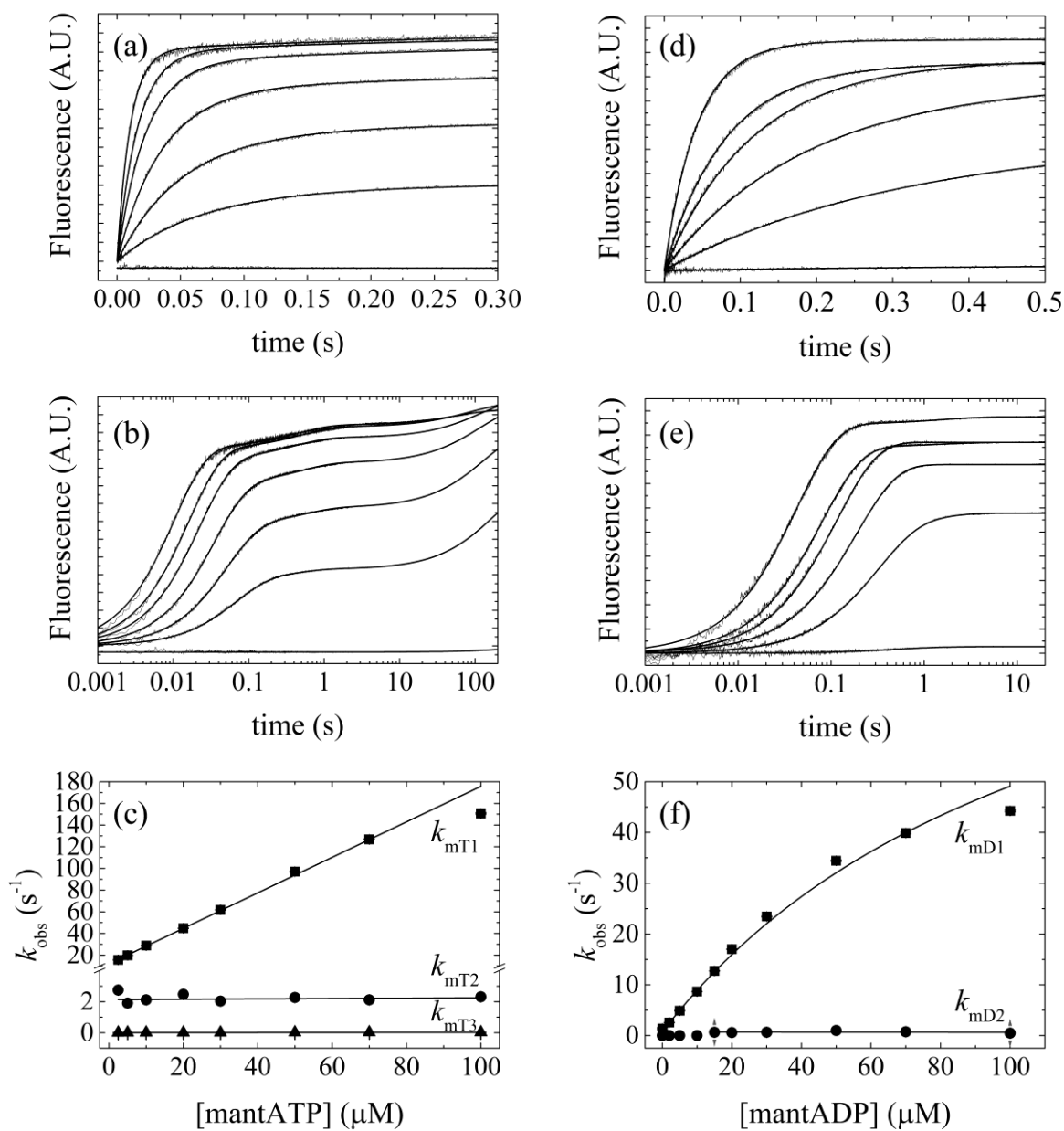
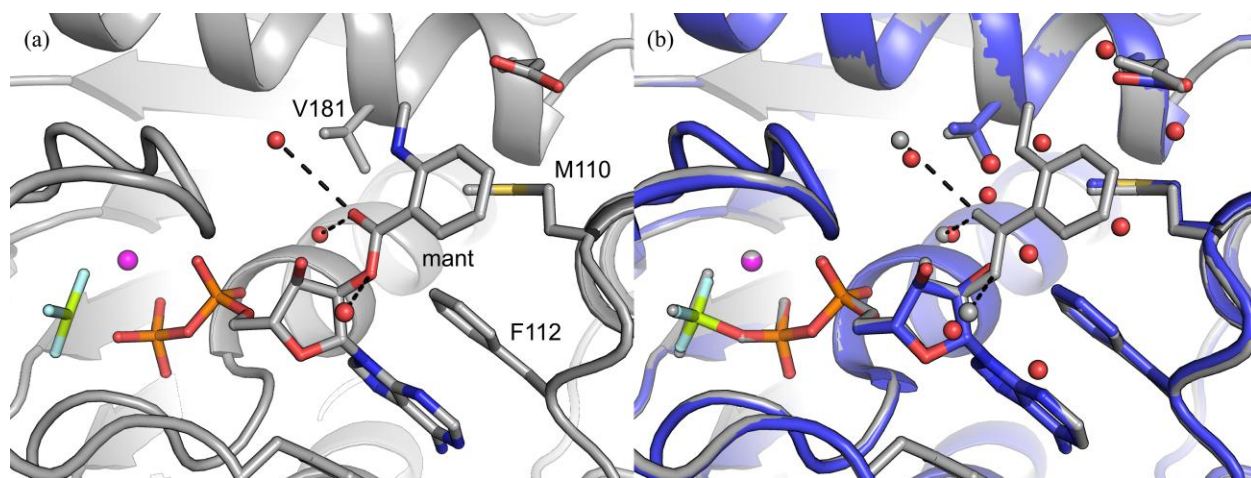


Figure 2



**Figure 3**

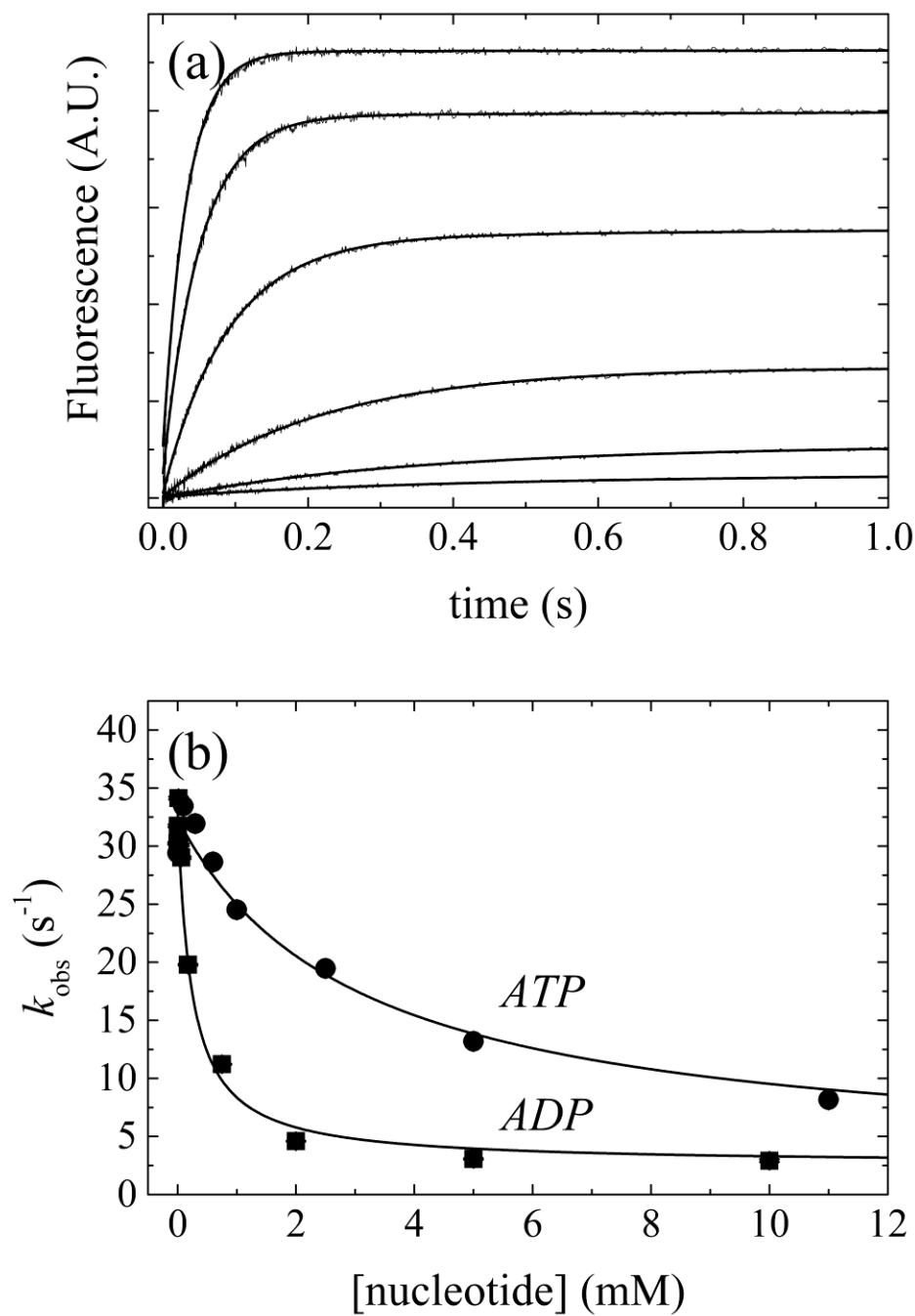
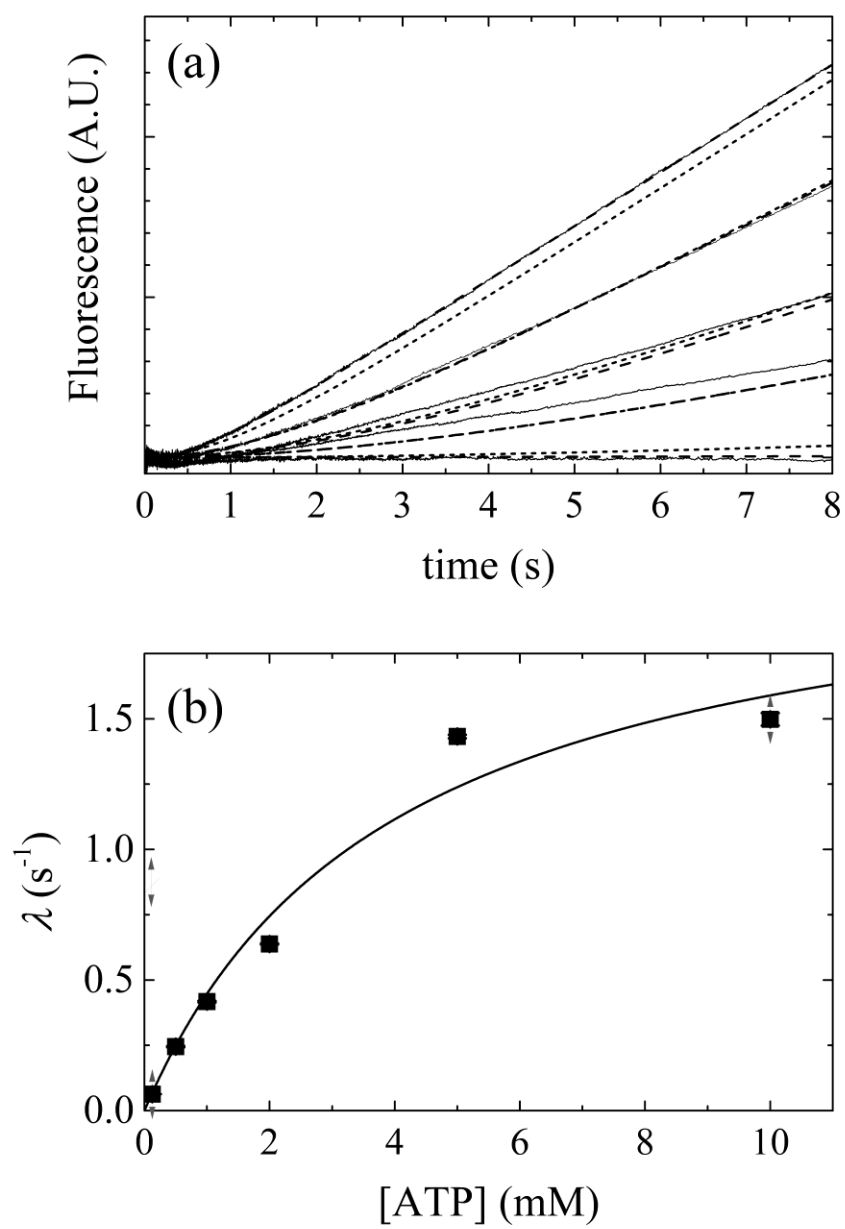
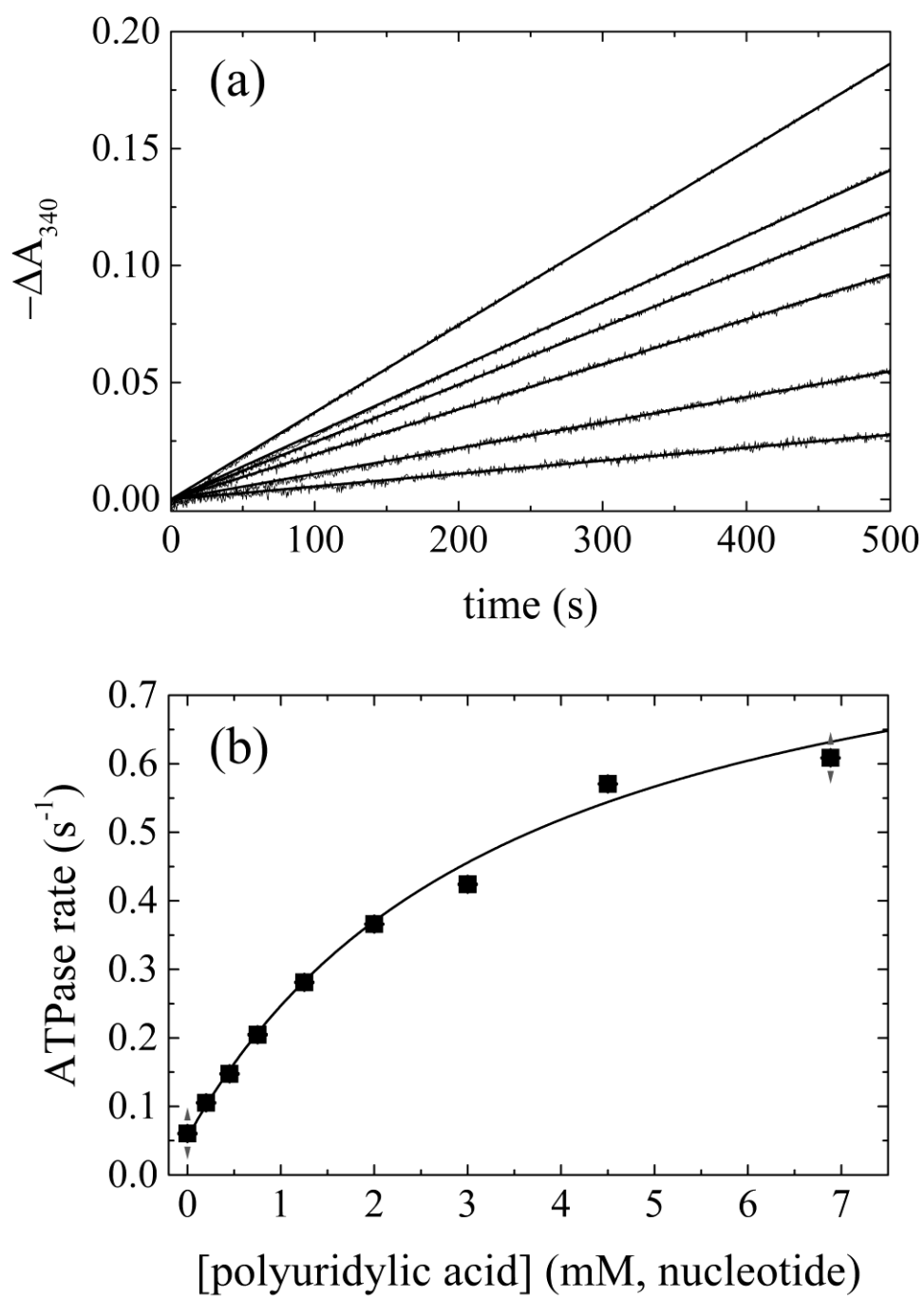


Figure 4

**Figure 5**

**Figure 6**

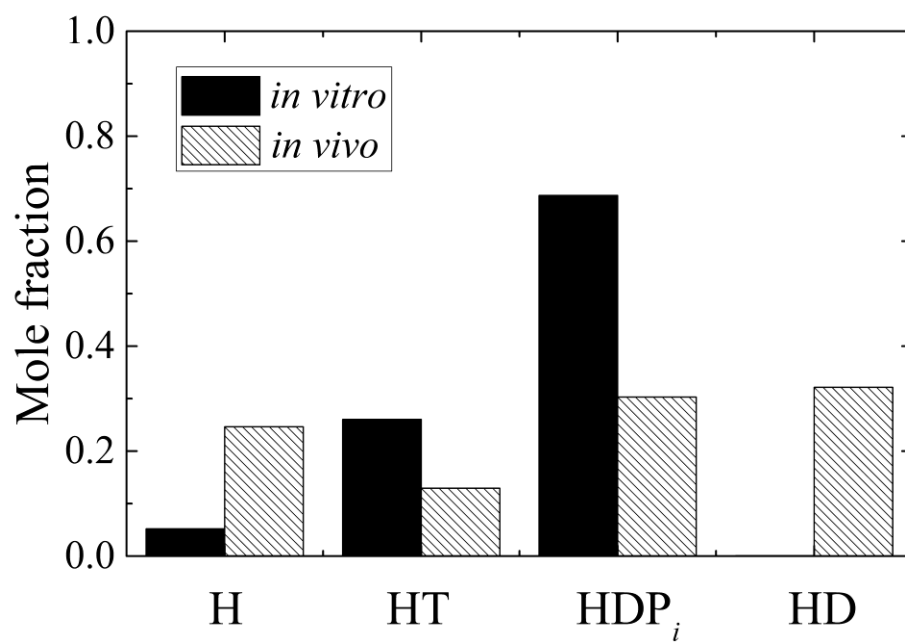
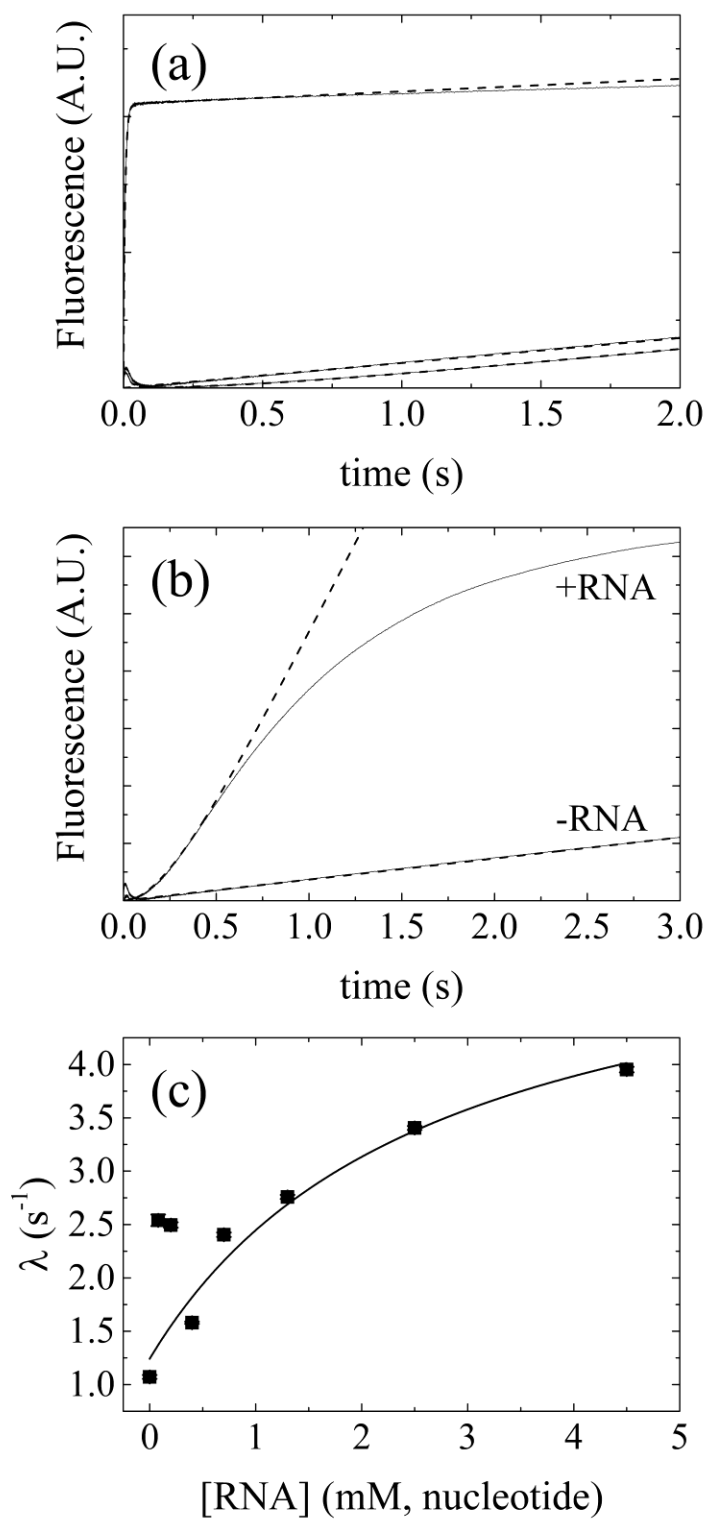
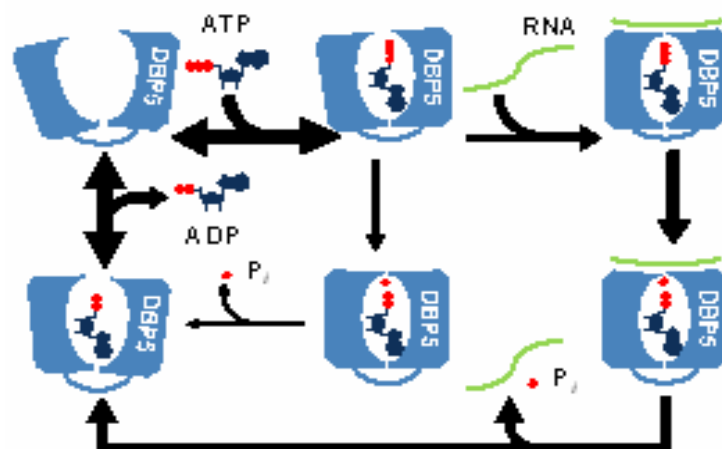


Figure 7

**Figure 8**



Graphical abstract



**Highlights**

- mRNA export from the nucleus requires DEAD-box protein Dbp5/DDX19 ATPase activity
- Kinetics and thermodynamics of intrinsic Dbp5 ATPase reveal RNA's effect on Dbp5
- Intrinsic Dbp5 ATPase is limited by slow ATP hydrolysis and slower  $P_i$  release
- RNA activates  $P_i$  release, but it and RNA binding still limit RNA-stimulated ATPase
- RNA binding and  $P_i$  release define RNA-stimulated Dbp5 ATPase for further regulation